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# Slow fusion of liposomes composed of membrane-spanning lipids

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## Abstract

The fusion characteristics of large unilamellar liposomes composed of bipolar tetraether lipids extracted from the thermophilic archaeon *Sulfolobus acidocaldarius*, was investigated. These lipids span the entire membrane and form single monolayer liposomes in aqueous media [Elferink, M.G.L., de Wit, J.G., Demel, R., Driessen, A.J.M. and Konings, W.N., (1992) J. Biol. Chem. 267, 1375–1381]. In the presence of calcium-phosphate, slow mixing of the aqueous liposome contents and membrane lipids occurred, demonstrating that these liposomes are fusion-competent. The fusion process was essentially nonleaky. The rate of fusion increased with the pH and the concentration of calcium and phosphate. Fusion resulted in an increase of the size of the liposomes. These data demonstrate that a monolayer organization of lipids in a membrane does not per se interfere with membrane fusion competence. © 1997 Elsevier Science Ireland Ltd.

**Keywords:** Slow fusion; Liposomes; Membrane-spanning lipids

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## 1. Introduction

Membrane fusion is a critical event in a variety of cell-biological processes. Since the primary function of membranes is to maintain the integrity of cells within their environment and the organi-

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zation of subcellular compartments inside the cell, membrane fusion must be strictly controlled in space and time. Recent investigations of intracellular membrane trafficking and virus–cell interactions have revealed many aspects of the molecular mechanisms involved in the control of biological membrane fusion reactions (White, 1992). Yet, little is known about the physical reorganization of lipids occurring during the process of membrane merging per se.

Early work by Palade (Palade, 1975) revealed pentalaminar structures and trilaminar diaphragms at the contact sites of interacting membranes, suggestive of the involvement of a hemifusion intermediate in the process of membrane merging. In later morphological studies of fusing membranes, relying on modern rapid-freezing techniques, such hemifusion structures were not seen, the earlier observations on trilaminar diaphragms being attributed to fixation artefacts (Chandler and Heuser, 1980). Yet, recent investigation of influenza virus has provided convincing evidence to indicate that membrane fusion of this virus may well proceed via a hemifusion intermediate (Kemble et al., 1994; Melikyan et al., 1995), although, rather than expanding into a extensive area of contact, the hemifusion intermediate would rapidly develop into a localized fusion pore (Palade, 1975; Tse et al., 1993). The notion of a fusion mechanism involving a distinct hemifusion stage with formation of a subsequent pore ('stalk-pore' mechanism) is further supported by recent theoretical and experimental work of Chernomordik and coworkers (Chernomordik et al., 1995).

Formation of a hemifusion intermediate requires a temporal separation of the leaflets of the interacting membranes. In this perspective, we considered it of interest to investigate the fusion competence of lipid vesicles composed of membrane-spanning lipids. Membranes of the thermoacidophile *Sulfolobus acidocaldarius* contain 99.9% tetraether lipids, which are comprised of a mixture of diglyceroltetraethers and nonitolglyceroltetraethers (Langworthy and Pond, 1986). In such systems, where the vesicular membranes, in fact, consist of a monolayer of bipolar lipids, separation of two bilayer leaflets is not possible.

Here, we demonstrate that, nevertheless, such vesicles are capable of undergoing nonleaky fusion, be it that this fusion reaction can only be induced under extreme conditions and proceeds at a relatively slow rate.

## 2. Materials and methods

### 2.1. Materials

Pyridine-2,6-dicarboxylic acid (DPA) was obtained from Sigma (St. Louis, MO), and  $\text{TbCl}_3$  was from Aldrich (Bornem, Belgium). *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-dipalmitoyl-L- $\alpha$ -phosphatidylethanolamine (*N*-NBD-PE) was obtained from Molecular Probes (Eugene, OR), and *N*-(lissamine rhodamine B sulfonyl) diacyl-phosphatidylethanolamine (*N*-Rh-PE) was from Avanti Polar Lipids (Alabaster, AL). Tetraether lipids were isolated from freeze-dried cells of *S. acidocaldarius* (DSM 639) as described (Elferink et al., 1993), and stored in chloroform/methanol/water (65:25:4, v/v/v) at 4°C.

### 2.2. Liposomes

Lipids were dried by rotary evaporation and suspended at a concentration of 10 mg/ml in an appropriate buffer system. Lipids were dispersed by sonication using a probe-type sonicator (intervals of 15 s sonication and 45 s rest) at 0°C, and stored in liquid nitrogen. Before use, samples were slowly thawed at room temperature and extruded through Unipore polycarbonate filters (Avestin, Ottawa, Canada), with a pore size of 200 nm, using a small-volume extrusion apparatus (Liposofast™ Basic, Avestin) (MacDonald et al., 1991).

### 2.3. Fusion assays

Lipid mixing was measured with the resonance energy transfer (RET) fusion assay as described (Struck et al., 1981). Liposomes were formed with and without 0.6 mole% each of *N*-NBD-PE and *N*-Rh-PE, and suspended at 10 mg/ml in buffer A

(100 mM NaCl, 1 mM EDTA, 10 mM Na-Hepes, pH 7.4). The lipid concentration of the liposome preparations was determined by phosphate analysis using Malachite Green (Driessen et al., 1991), after destruction of the liposomes with 70%  $\text{HClO}_4$ . Labelled and nonlabelled liposomes were mixed at a one-to-one ratio, and diluted to a final lipid concentration of 50  $\mu\text{M}$  (lipid phosphorous) in buffer A. Fluorescence measurements were performed at 25°C using a Perkin Elmer LS-50B spectrophotofluorimeter using an excitation and emission wavelength of 465 and 535 nm, respectively. A 520 nm cutoff filter was used in the emission path to eliminate light scattering.  $\text{Ca}^{2+}$  was added to sample and, after 2 min, fusion was initiated by the addition of phosphate to the indicated concentrations. The maximal extent of fusion was determined by subjecting an undiluted one-to-one mixture of labelled and unlabelled liposomes to two cycles of freezing and thawing. This treatment proved to be sufficient for obtaining complete lipid mixing, and further cycles had little effect on the fluorescence level. Fused liposomes were diluted to 50  $\mu\text{M}$ , and the fluorescence level of this suspension was set to 100%.

Mixing of aqueous contents of the liposomes was determined with the Tb/DPA fusion assay (Wilschut and Papahadjopoulos, 1979; Wilschut et al., 1980). Liposomes were prepared, as described above, at a concentration of 10 mg lipid/ml in buffer B (5 mM  $\text{TbCl}_3$ , 50 mM Na-citrate, 10 mM Na-Hepes, pH 7.4), buffer C (50 mM DPA, 20 mM NaCl, 10 mM Na-Hepes pH 7.4), or buffer D (2.5 mM  $\text{TbCl}_3$ , 25 mM Na-citrate, 25 mM DPA, 10 mM NaCl, 10 mM Na-Hepes, pH 7.4), and are referred to as 'Tb-loaded', 'DPA-loaded' and 'Tb/DPA-loaded' liposomes, respectively. After extrusion, the nonencapsulated material was removed by gel filtration of the liposomes on Sephadex G-25M columns PD-10 (Pharmacia, Uppsala, Sweden) in buffer A. Tb- and DPA-loaded liposomes were mixed at a one-to-one ratio in buffer A at a final lipid concentration of 50  $\mu\text{M}$ . The formation of the Tb/DPA complex was measured with an SLM-4000 spectrophotofluorimeter using excitation and emission wavelengths of 276 and 545 nm, respectively. A 530 nm cutoff filter was placed in the emission

path to eliminate possible contributions of light scattering to the signal. Our instrument has a 'T' format design which allows the simultaneous registration of fluorescence and 90° light scattering. Light scattering was measured in the second emission channel at 430 nm wavelength. The temperature of the sample holder was maintained at 25°C, and the solution in the cuvette was continuously stirred. Fusion was induced as described for the RET assay. Tb/DPA-loaded liposomes were used to calibrate the signal to 100% fusion, and to determine liposome leakage under the conditions of the fusion experiment.

#### 2.4. Electron microscopy

Cryo transmission electron microscopy was performed using a Philips CM 10 electron microscope with a liquid nitrogen-cooled gatan cryo device. Sample preparation involved liquid propane fast cooling.

### 3. Results and discussion

Mixing of membrane lipids was measured with the resonance energy transfer (RET) assay. To ensure that the RET probes mix appropriately with the tetraether lipids a calibration curve was constructed in which the percentage of *N*-NBD-PE fluorescence was plotted as a function of the mol% *N*-NBD-PE and *N*-Rh-PE. *N*-NBD-PE fluorescence in the absence of *N*-Rh-PE was set at 100%. The straight line observed at the lower probe concentrations indicated that distribution of the probes was random. For the fusion experiments liposomes were prepared of tetraether lipids and 0.6 mol % of *N*-NBD-PE and *N*-Rh-PE. Labelled liposomes were mixed with an equal quantity of non-labelled liposomes. Upon fusion, the fluorophores dilute into the plane of the non-labelled liposomes resulting in a decrease in energy transfer efficiency. This can either be measured as an increase in the fluorescence of the energy donor NBD, or a decrease of the fluorescence of the acceptor rhodamine. We monitored the change in the NBD fluorescence intensity. In the presence of calcium and phosphate, a slow

increase in the NBD fluorescence was evident (Fig. 1). This suggests that calcium/phosphate induces fusion and intermixing of membrane lipids among the liposomes composed of tetraether lipids. This process is, however, slow as compared to calcium/phosphate-induced fusion of liposomes composed of acidic phospholipids. Under the conditions employed, it appeared that the fusion reaction was not completed after 110 min.

To ensure that mixing of lipids, as detected with the RET assay, was the result of a bona fide membrane fusion process and not due to just probe transfer, fusion was also measured with the Tb/DPA assay. This method monitors the mixing of aqueous liposomal contents. For this purpose, one population of liposomes was loaded with terbium and the other population was loaded with DPA. Fusion of the liposomes results in the for-

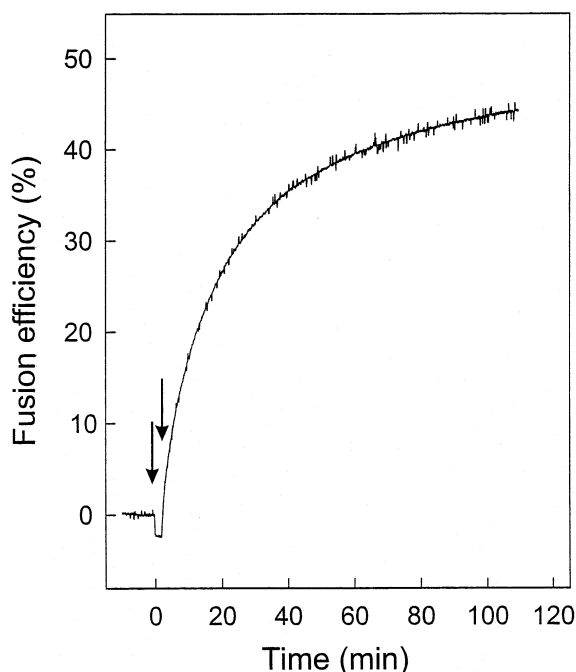


Fig. 1. Mixing of membrane lipids during the calcium-phosphate induced fusion of liposomes composed of tetraether lipids. Liposomes labelled and nonlabelled with 0.6 mol % of *N*-NBD-PE and *N*-Rh-PE were mixed at a one-to-one ratio at a lipid concentration of 50  $\mu$ M in buffer A. Fusion was initiated, 2 min after the addition of CaCl<sub>2</sub> to a final concentration of 4.5 mM (first arrow), by the addition of potassium phosphate to a concentration of 10 mM (second arrow).

mation of the fluorescent Tb/DPA complex, while a low concentration of the chelator EDTA in the external medium, even in the presence of an excess of calcium, prevents the formation of the complex outside the liposomes. In the presence of calcium and phosphate, a slow increase in the Tb fluorescence occurred, indicative for fusion of the liposomes (Fig. 2A, trace *a*). Fusion proceeded during 110 min up to 38%, with kinetics very similar to the kinetics observed in the lipid mixing experiment (Fig. 1). No signal was observed when liposomes were used that were not loaded with Tb or DPA (trace *b*).

Importantly, the calcium/phosphate-induced fusion process was found to be completely non-leaky. Leakage of the liposomal contents was measured with liposomes pre-loaded with the Tb/DPA complex. Fig. 2B shows that there was essentially no leakage of the Tb/DPA complex to the external medium during the first 80 min of the fusion process. At approximately 110 min, the fluorescence level suddenly dropped to lower values. This phenomenon was accompanied with a large increase in the light scattering properties of the sample (Fig. 2C), and has also been observed in studies of calcium/phosphate-induced fusion of acidic phospholipid vesicles (Fraley et al., 1980). It is associated with the conversion of amorphous calcium/phosphate (ACP) into octacalciumphosphate (OCP) and subsequently into hydroapatite (HAP). During this conversion, a thick precipitate appeared in the cuvette that floated on top of the surface and stuck to the glass walls. The drop in fluorescence level was only to a limited extent caused by release of the Tb/DPA complex to the medium. It was primarily due to clearance of the aggregated liposomes from the light beam, since rigorous shaking of the cuvette largely recovered the fluorescence signal (Fig. A and B, broken line). A similar effect was observed when an excess of EDTA (20 mM) was added to the suspension to dissolve the calcium/phosphate precipitates (not shown). This indicates that during the conversion of ACP via OCP to HAP the contents of the fused liposomes is largely retained.

The calcium/phosphate-induced fusion of the tetraether liposomes, as followed by the Tb/DPA assay, was further characterized by varying the

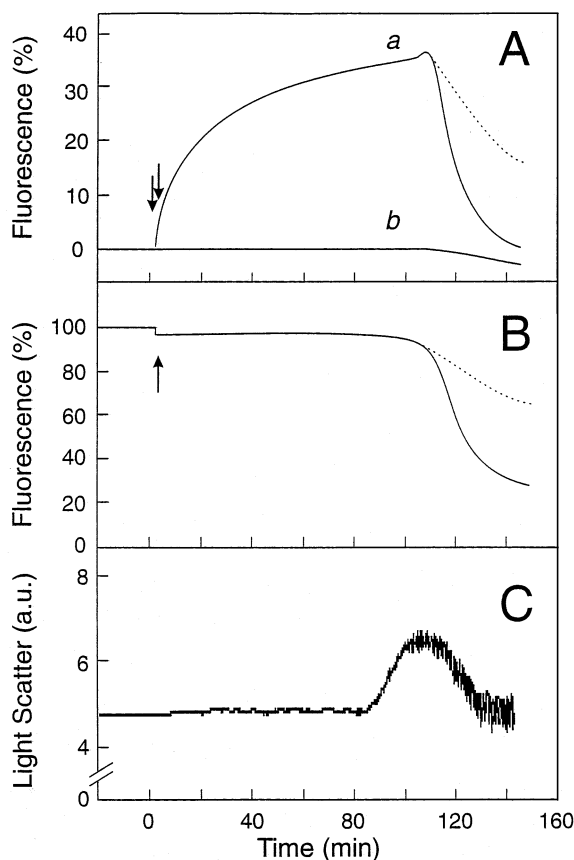


Fig. 2. Mixing of aqueous contents (A), leakage (B) and light scatter (C) during the calcium-phosphate induced fusion of liposomes composed of tetraether lipids. A. Tb-loaded liposomes were mixed at a one-to-one ratio with DPA-loaded liposomes at a lipid concentration of  $50 \mu\text{M}$  in buffer A (trace a). Fusion was initiated, 2 min after the addition of  $\text{CaCl}_2$  to a final concentration of  $4.5 \text{ mM}$  (arrow 1), by the addition of potassium phosphate to a concentration of  $10 \text{ mM}$  (arrow 2). Fusion was followed fluorometrically by monitoring the formation of the Tb/DPA complex (solid line). The potential contribution of light-scattering to the fluorescence signal was checked by following the fusion of liposomes that were not loaded with Tb and DPA (trace b). B. Leakage of aqueous contents was measured by the use of liposomes loaded with the Tb/DPA complex. The loss of the fluorescent signal after 110 min is largely due to precipitation of the fused liposomes. Manual shaking of the cuvette recovered most of the signal (broken lines). C. Light-scattering was measured at  $430 \text{ nm}$  at  $90^\circ$  incidence of the excitation beam.

concentration of  $\text{Ca}^{2+}$  and phosphate, and by changing the pH of the suspension (Fig. 3). The rate at which calcium/phosphate converts from

ACP to HAP determines in part the kinetics of vesicle fusion and leakage in a calcium/phosphate-containing medium, and the kinetics of the calcium/phosphate conversions, in turn, are strongly dependent of the relative concentrations of calcium and phosphate and on the pH. Fusion was strictly  $\text{Ca}^{2+}$  dependent (Fig. 3A). In the absence of phosphate, very slow fusion of the liposomes occurred in the presence of high  $\text{Ca}^{2+}$  concentrations, i.e.  $\geq 20 \text{ mM}$  (data not shown). High phosphate concentrations, i.e.  $> 15 \text{ mM}$ , favour the formation of the hydroxyapatite complex, and caused the rapid formation of aggregates and leakage. A similar phenomenon was observed when the pH was increased to values above pH 7.4. An increase in the temperature resulted in apparent lower fusion rates. For instance, at  $50^\circ\text{C}$ , already 3 min after the addition of phosphate the formation of a thick precipitate was evident. This made the liposomes almost completely leaky.

The liposomes fused upon calcium/phosphate addition were examined with cryo transmission electronmicroscopy. For this purpose, it was necessary to use a lipid concentration that was at least four-fold higher than that used in the fluorescence fusion assays. Under these conditions, also a high concentration of  $\text{Ca}^{2+}$  is required to induce fusion. By following the light scatter properties of the liposomal suspension it was established that the time course of fusion was almost identical to the one shown in Fig. 2, when  $7.5 \text{ mM}$  of  $\text{Ca}^{2+}$  was used instead of  $4.5 \text{ mM}$ . The electron micrographs show that the liposomes increased in size when exposed to calcium/phosphate for increasing periods of time (Fig. 4A,B). At the later stage of the fusion, extensive aggregation of the liposomes was evident (Fig. 4C).

This study demonstrates that liposomes composed of bipolar membrane-spanning lipids have the ability to undergo membrane fusion, as evidenced by the occurrence of mixing of the lipids of the interacting vesicles and nonleaky coalescence of their aqueous volumes. Fusion could only be induced by calcium/phosphate and it proceeded at a comparatively slow rate. Calcium/phosphate-induced fusion has also been observed for anionic phospholipid vesicles, composed of

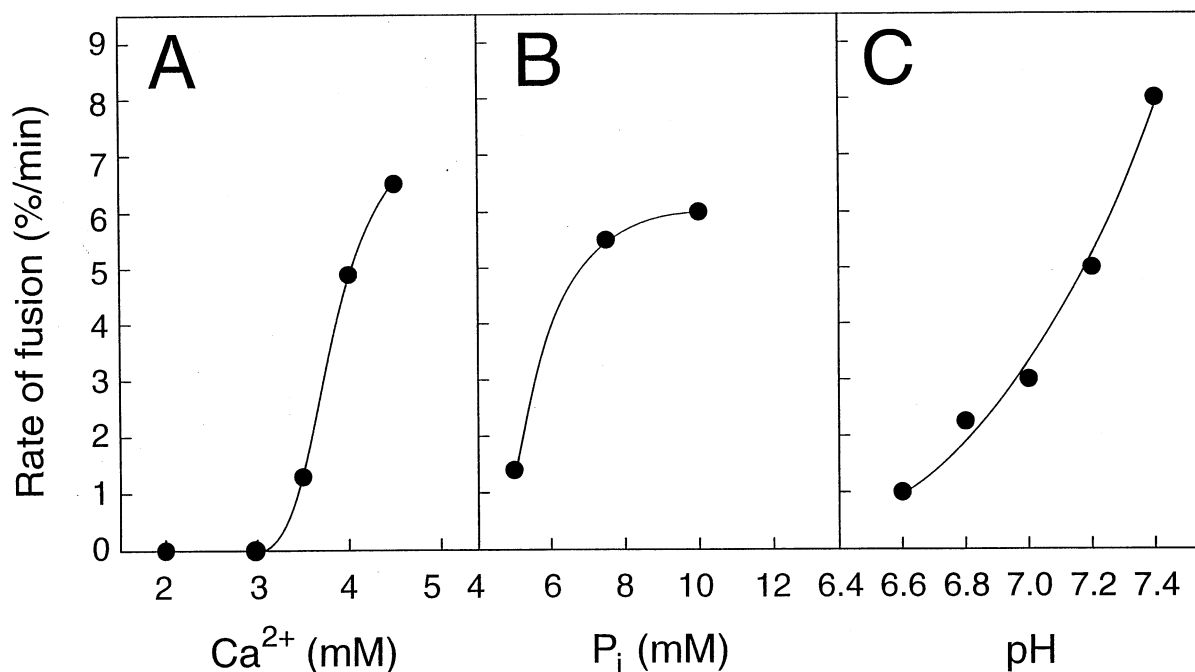


Fig. 3.  $\text{Ca}^{2+}$  (A), phosphate (B), and pH (C) dependency of fusion of liposomes composed of tetraether lipids. Fusion was measured with the Tb/DPA assay, as described in Section 2 and in the legend to Fig. 2. The assay conditions used were: 4.5 mM  $\text{CaCl}_2$ , 10 mM potassium phosphate, pH of 7.4, unless indicated otherwise.

phosphatidylserine and cholesterol (Fraley et al., 1980), and for erythrocyte ghosts (Hoekstra et al., 1983). In these earlier investigations, like in the present study, the rates of fusion and vesicle leakage were strongly dependent on the relative concentrations of calcium and phosphate, the pH, and the temperature, each affecting the rate of conversion of the calcium/phosphate complex from ACP to HAP. It is not clear how calcium/phosphate mediates membrane fusion. The initial complex, formed upon addition of phosphate to calcium, presumably establishes close contact between the interacting membranes, the subsequent conversions in the complex inducing membrane merging and ultimate lysis when HAP is formed. Irrespective of the precise mechanism, it is interesting that membranes consisting of bipolar lipids fuse at all under these conditions. Freeze-fracturing experiments show that there is no fracture plane to break the membranes into two leaflets, rather cross-fracturing of the whole membrane was observed (Elferink et al., 1992). This is consis-

tent with a monolayer organization of the membrane. Accordingly, it is very unlikely that the fusion process in this system involves temporal separation of membrane leaflets. This implies that membranes can fuse through a mechanism that does not involve a distinct hemifusion intermediate. Whether or not this is of physiological relevance remains to be seen. Perhaps the slow rate of fusion seen in our present study, as compared to the faster fusion of anionic phospholipid vesicles (Fraley et al., 1980) or erythrocyte ghosts (Hoekstra et al., 1983) in the presence of calcium/phosphate reflects the large energy barrier involved in fusion of membranes composed of bipolar lipids. This would argue in favour of fusion of 'normal' bilayer membranes composed of monopolar lipids through a process that does involve transient separation of the two bilayer leaflets and the formation of a hemifusion intermediate structure. Yet we feel it is intriguing, that in principle, membranes may have access to alternative mechanisms of fusion.

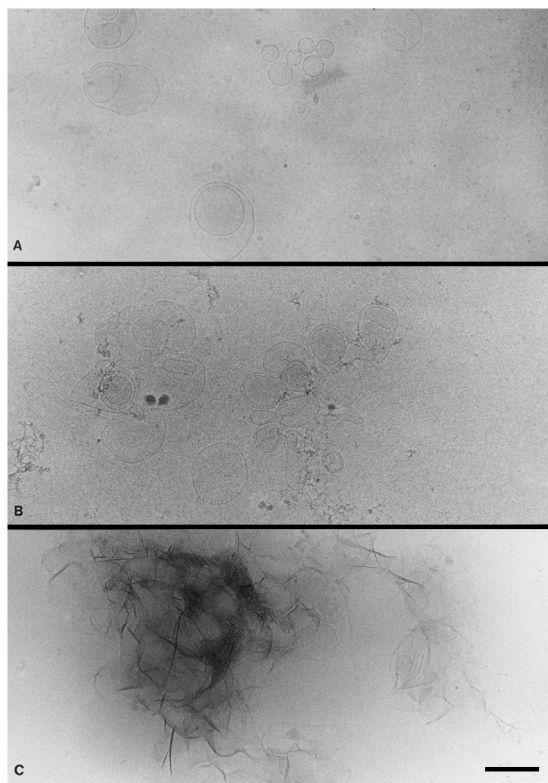


Fig. 4. Cryo transmission electron microscopical images of different stages of the calcium-phosphate induced fusion of liposomes composed of tetraether lipids. Liposomes were suspended in buffer A at a lipid concentration of 200  $\mu$ M. Fusion was induced by the addition of  $\text{CaCl}_2$  and potassium phosphate (pH 7.4) to final concentrations of 7.5 mM and 10 mM, respectively. The images represent 0 (A), 60 (B), and 120 (C) min time points. Bar represents 0.2  $\mu$ m.

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